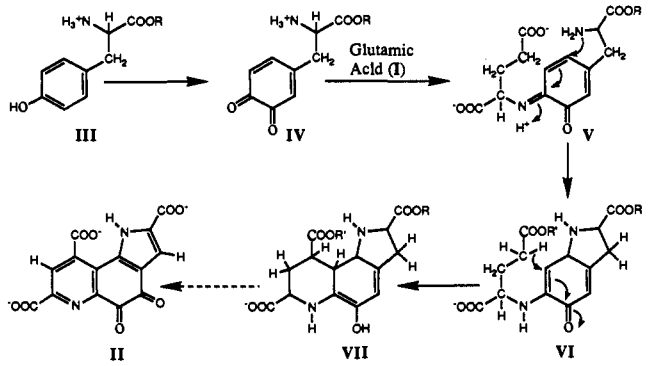
Scheme II



Labeling with [α-^{15}N,2',6'-$^{13}C_2$]Tyrosine. To determine if the pyrrole nitrogen was derived from the α-amino group of tyrosine, L-[α-^{15}N,2',6'-$^{13}C_2$]tyrosine was fed to *M. extorquens* AM1 cultures. Intramolecular cyclization of this compound would yield PQQ with a ^{15}N (N-1) directly bonded to ^{13}C (C-1a) and would result in a direct ^{13}C–^{15}N spin–spin coupling network. The spectrum of PQQ isolated from this culture was labeled with ^{13}C at C-4 and C-1a; the ^{13}C enrichment, estimated from the ^{1}H NMR of signal of H-3, is 50.7%. From integration of the ^{13}C NMR signal of C-1a (Figure 4), it is estimated that 15.7% of the molecules labeled with ^{13}C also contain ^{15}N ($^1J_{C\text{-}1a\text{-}N}$ = 14.8 Hz). The overall ^{15}N enrichment was determined to be 10.6% from integration of ^{1}H NMR signal of the N–H resonance, which is directly coupled to N-1 ($^1J_{N\text{-}H}$ = 97 Hz). As expected, there is significant dilution of the ^{15}N label due to transamination; however, the incorporation of ^{15}N into PQQ is 30-fold greater than that expected if the tyrosine nitrogen equilibrated with the free ammonium pool. In addition, there is a strong correlation between ^{13}C and ^{15}N labeling. That is, of the PQQ molecules labeled with ^{15}N in the pyrrole nitrogen, a greater fraction contains ^{13}C at C-1a (75%) than contains ^{12}C at C-1a (25%). Clearly the α-nitrogen of tyrosine is not randomized in PQQ, demonstrating that tyrosine, and not its requisite α-keto acid, is the precursor for PQQ biosynthesis.

Conclusion

The data presented here demonstrate that PQQ is biosynthesized from the amino acids tyrosine and glutamate. In contrast to the biosynthetic pathways leading to other cofactors such as riboflavin or folic acid, the biochemical transformation that leads to PQQ is remarkably efficient in the sense that all the carbons and probably both nitrogens of the precursors are conserved in the product. Because the process involves the loss of 12 electrons in the conversion of tyrosine and glutamate to product, PQQ biosynthesis involves primarily oxidative reactions. Oxidation of the phenol side chain must be an early step in the pathway because it is requisite for the formation of the pyrrole ring by cyclization of the tyrosine backbone. Given our data, we have outlined a possible route for PQQ biosynthesis (Scheme II). In this route, tyrosine (III) or some derivative of tyrosine is oxidized to dopaquinone (IV) in a reaction catalyzed by a monophenol monooxygenase like enzyme (tyrosinase, EC 1.14.18.1). Glutamate (I) could form a Schiff base with dopaquinone. The cyclization of the tyrosine backbone to form the pyrrole ring could occur by a Michael-type addition analogous to the known nonenzymatic cyclization of dopaquinone to form dopachrome.[27] Alternatively, the biosynthesis of PQQ may involve dopachrome as an intermediate to which glutamate is added in a subsequent reaction. To date, we have been unable to detect tyrosinase activity in crude extracts of *M. extorquens* AM1. In addition, no sequence homology exists between tyrosinase genes from *Streptomyces glaucescens*[29] or *Neurospora crassa*[30] and the PQQ biosynthesis genes examined to date from *Acinetobacter calcoaceticus*.[31] The absence of tyrosinase activity may indicate that a derivatization of tyrosine precedes oxidation of its phenol side chain. Alternatively, the enzymatic oxidation of tyrosine may donate electrons to an intermediate redox cofactor rather than directly to O_2.

Acknowledgment. This work was supported by the DOE, OBES, Division of Biological Energy Research and by the National Stable Isotopes Resource NIH (RR 02231).

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How Can the Solvent Affect Enzyme Enantioselectivity?

Paul A. Fitzpatrick and Alexander M. Klibanov*

Contribution from the Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139. *Received October 5, 1990*

Abstract: Enantioselectivity of the protease subtilisin Carlsberg in the transesterification between the chiral alcohol *sec*-phenethyl alcohol and vinyl butyrate was found to be greatly affected by the solvent. For example, the $(k_{cat}/K_M)_S/(k_{cat}/K_M)_R$ ratio varies from 3 in anhydrous acetonitrile to 61 in anhydrous dioxane. A mechanistic model is proposed that explains these findings. This model is supported by the experimental data obtained concerning the dependence of subtilisin's enantioselectivity on the structure of the chiral alcohol, on physicochemical characteristics of the solvent (systematic correlations were found with the dielectric constant and the dipole moment), and on such additives as water and the water mimic formamide. Similar dependencies (although of a smaller magnitude) were observed for the related enzyme subtilisin BPN'.

To organic chemists, enantioselectivity is the most valuable feature of enzymes.[1] A major obstacle to a wider synthetic exploitation of enzyme enantioselectivity is its relative inflexibility: under each set of conditions, the stereochemical outcome of a given enzyme–substrate reaction is predetermined. If one wishes to alter it, two options are available—to change the reactants or to change the reaction conditions. The former, although frequently used, requires either an (time-consuming and empirical) enzyme screening or a (otherwise unnecessary and often undesirable) deviation from the initial substrate structure.[1] Thus, in principle, the latter option is preferable; there are a few recent illustrations of it, e.g., the temperature dependence of enantioselectivity of some

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thermophilic alcohol dehydrogenases.[2] Also, there have been reports of appreciably improving the optical purity of the products of enzymatic resolutions of racemates by small fractions of water-miscible cosolvents.[3]

The advent of enzymatic catalysis in neat organic solvents has allowed variation, in a fundamental and systematic way, of the reaction medium of enzymatic processes, thereby bringing about striking alterations in enzyme properties.[4] In particular, it has been demonstrated that enantioselectivity of enzymes can be greatly affected by the nature of the solvent[5,6] and its water content.[7] Our ultimate goal is to control rationally and to be able to predict enantioselectivity of enzymes as a function of the solvent. This undertaking, if successful, will lead to enzyme enantioselectivity "to order" by means of simply switching from one reaction medium to another.

Several years ago, we introduced a new strategy for the preparative resolution of racemic alcohols catalyzed by hydrolytic enzymes.[8] Instead of using the conventional enzymatic hydrolysis in water of an ester of this alcohol and an achiral acid,[1] we employed this chiral alcohol as a nucleophile in an enzymatic transesterification of an achiral ester in organic solvents containing little or no water. Since then, this strategy, with various lipases and proteases as catalysts, has been profitably utilized in dozens of studies and expanded to other classes of compounds and asymmetric processes.[9] In the present work, we investigated such an enzymatic transesterification to answer the title question.

Results and Discussion

We selected subtilisin Carlsberg (serine protease from *Bacillus licheniformis*) as a transesterification catalyst in anhydrous organic solvents for the following reasons: (i) this enzyme, available in a highly purified form, is catalytically active in a number of anhydrous solvents, and its behavior in such media has been thoroughly investigated;[10] (ii) by means of Hammett analysis and kinetic isotope effect examination, the mechanism of subtilisin action has been found to be the same in water and in various anhydrous solvents[11] (furthermore, a solid-state NMR study revealed that the structures of the catalytic site of a sister serine protease, α-lytic protease from *Lysobacter enzymogenes*, in water and in anhydrous acetone and octane are indistinguishable[12]); and (iii) subtilisin's enantioselectivity in the transesterification reaction between *N*-acetylalanine and propanol has been found to be dependent on the solvent;[5] hence, it should be of interest to compare this phenomenon with the data obtained in the present work.

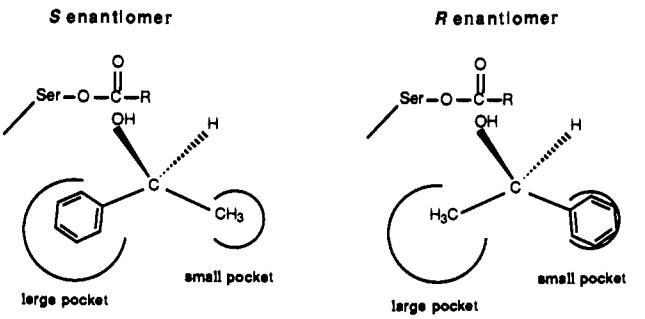

Figure 1. Schematic representation of the modes of binding of *S* and *R* enantiomers of **1** to the binding site of acyl-subtilisin (in the present paper, R = C_3H_7). Ser refers to the serine-221 residue, which is a part of the catalytic triad and the head nucleophile of subtilisin. See text for details.

Table I. Enantioselectivity of Subtilisin Carlsberg in the Transesterification between Vinyl Butyrate and Homologous Chiral Alcohols in Anhydrous Solvents[a]

	enantioselectivity,[18] v_S/v_R		
alcohol	dioxane[b]	acetonitrile	tetrahydrofuran
sec-phenethyl alcohol	50	3.6	34
sec-(2-naphthyl)ethyl alcohol	58	20	56
sec-octanol	100	14	80
sec-butanol	3.9	1.2	3.2
2-chloro-*sec*-phenethyl alcohol	6.8	2.1	5.6

[a] The initial rates for individual enantiomers of the chiral alcohols listed (v_S and v_R) were measured at 1 mg/mL enzyme, 10 mM alcohol, and 200 mM vinyl butyrate; for other conditions and procedures, see the Experimental Section. No reaction was observed without enzyme. [b] Dioxane here and hereafter stands for 1,4-dioxane.

Initially, we studied the kinetics of the transesterification reaction between the chiral alcohol *sec*-phenethyl alcohol[13] (**1**) and the activated ester vinyl butyrate[14] catalyzed by subtilisin in anhydrous dioxane:[15]

$$PhCH(CH_3)OH + CH_3CH_2CH_2COOCH{=}CH_2 \rightarrow CH_3CH_2CH_2COOCH(CH_3)Ph \quad (1)$$

Powdered subtilisin was suspended[10] (1 mg/mL) in dioxane containing 10 mM **1** (*R* or *S* enantiomer) and 200 mM vinyl butyrate. Following a brief sonication, the mixture was shaken at 45 °C and 300 rpm; periodically aliquots were withdrawn and analyzed by gas chromatography. The initial rates for *S* and *R* enantiomers (v_S and v_R, respectively) of **1** were determined to be 1.1 and 0.022 mM/h, respectively; i.e., the *S* enantiomer was 50-fold more reactive toward subtilisin than its *R* counterpart. The enantioselectivity was even greater, 61 times, when k_{cat}/K_M values for the enantiomers of **1** were compared.

Similarly to the classical mechanisms of enzyme enantioselectivity,[16] the observed phenomenon can be rationalized in terms of the model depicted in Figure 1. The model predicates that the nucleophile-binding site of subtilisin contains two pockets—large and small. The *S* enantiomer of **1** binds to the enzyme in a fashion shown in the left part of Figure 1; i.e., the phenyl group occupies the large pocket, and the methyl group the small one. This arrangement positions **1**'s hydroxyl group properly aligned to attack the carbonyl of butyryl-subtilisin.[17] When the phenyl

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(15) This solvent has been successfully used as the reaction medium for subtilisin-catalyzed reactions before.[10]

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Table II. Enantioselectivity of Subtilisin Carlsberg in Transesterification 1 in Various Anhydrous Solvents[a]

solvent[b]	k_{cat}/K_M, M^{-1} min^{-1}		enantioselect,[18]
	S enant	R enant	$(k_{cat}/K_M)_S/(k_{cat}/K_M)_R$
dioxane	170	2.8	61
benzene	13	0.24	54
triethylamine	330	6.9	48
tetrahydrofuran	230	5.8	40
pyridine	43	1.4	31
dimethylformamide	1.4	0.16	9
nitromethane	16	3.3	5
methylacetamide	1.3	0.38	3
acetonitrile	48	16	3

[a] The k_{cat}/K_M values for the individual enantiomers of **1** were determined as described in the Experimental Section under the following conditions: 1 mg/mL subtilisin, 200 mM vinyl butyrate; concentrations of **1** were varied between 10 and 70 mM. No transesterification was observed without enzyme. [b] Dioxane stands for 1,4-dioxane, and methylacetamide for *N*-methylacetamide.

and methyl substituents at the chiral carbon of the *R* enantiomer of **1** bind to the acyl-enzyme the same way as those of its *S* counterpart, then the hydroxyl group faces away from the carbonyl moiety, thereby making the nucleophilic attack impossible. The mode of binding of the *R* enantiomer conducive to the deacylation reaction is shown in the right portion of Figure 1, which illustrates that the phenyl group experiences severe steric hindrances in fitting into the small binding pocket. Consequently, the concentration of the reactive complexes for the *R* enantiomer should be much lower than for the *S* enantiomer, which would explain why the latter is so much more reactive in transesterification 1.

The model presented in Figure 1 affords several predictions. If the phenyl substituent in **1** is replaced by a bulkier group, then one would expect the enzyme enantioselectivity in dioxane to rise because the steric difficulties encountered by the *R* enantiomer will be exacerbated even further. Conversely, if the phenyl substituent is replaced with a smaller group, then the enantioselectivity of subtilisin should drop because it should become easier for the *R* enantiomer to bind to the acyl-enzyme in a productive fashion. In addition, if the methyl substituent of **1**'s chiral carbon is replaced with a bulkier group, then the enzyme enantioselectivity is expected to decrease because the *S* enantiomer will start having steric problems, while the productive binding of its *R* counterpart will be unaffected. These predictions, and hence the validity of the model, were verified experimentally. As seen in the first data column of Table I, upon transition from **1** as the nucleophile in reaction 1 to *sec*-(2-naphthyl)ethyl alcohol and *sec*-octanol, the enantioselectivity[18] of subtilisin increases from 50 to 58 and 100,

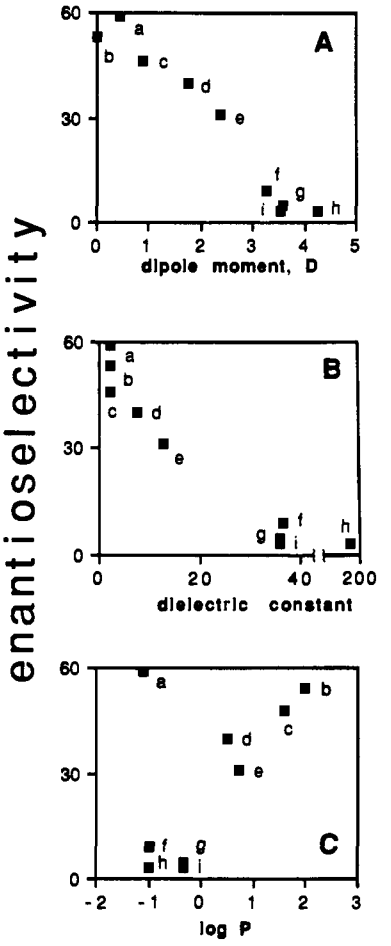

Figure 2. Enantioselectivity of subtilisin Carlsberg in transesterification 1 in anhydrous solvents as a function of the dipole moment (A), dielectric constant (B), and hydrophobicity (C) of the solvent. The values for dipole moments and dielectric constants were taken from ref 20 (pp 408–410). Solvent hydrophobicity values, defined as log *P* where *P* is a partition coefficient for a given solvent between n-octanol and water, were taken from ref 21. Enantioselectivity here[18] is defined as $(k_{cat}/K_M)_S/(k_{cat}/K_M)_R$, where *S* and *R* refer to the enantiomers of **1**. Solvents: a, 1,4-dioxane; b, benzene; c, triethylamine; d, tetrahydrofuran; e, pyridine; f, dimethylformamide; g, nitromethane; h, *N*-methylacetamide; i, acetonitrile. Experimental conditions are described in Table II.

respectively, whereas when *sec*-butanol is used instead, the enantioselectivity decreases from 50 to 3.9 (for the last three alcohols, v_S were 1.1, 0.21, and 0.59 mM/min, and v_R were 0.019, 0.0021, and 0.15 mM/min, respectively). Finally, when **1** is replaced with 2-chloro-**1**, the enantioselectivity drops from 50 to 6.8 (almost exclusively at the expense of the more reactive enantiomer: for 2-chloro-**1**, v_S = 0.17 mM/h and v_R = 0.025 mM/h).

The next step was to investigate the dependence of subtilisin's enantioselectivity in transesterification 1 on the reaction medium. To this end, k_{cat}/K_M values for *S* and *R* enantiomers of **1** were determined in nine unrelated anhydrous solvents and are presented in Table II in the order of descending enantioselectivity.[18] One can see that the solvent markedly affects the enantioselectivity of subtilisin (under otherwise identical conditions): for instance, upon transition from dioxane to acetonitrile as the reaction medium, $(k_{cat}/K_M)_S(k_{cat}/K_M)_R$ drops more than 20-fold.

To rationalize this phenomenon, it was important to ascertain which enantiomer of **1**, *S* or *R*, is primarily affected by the solvent thus causing the observed differences in enantioselectivities. The data in Table II do not directly furnish an answer to this question

(17) It has been previously established[10] that subtilisin-catalyzed transesterifications in organic solvents follow the compulsory order scheme without ternary complexes. This mechanism involves formation of a noncovalent enzyme–ester complex, followed by its transformation to an acyl-subtilisin intermediate with the concomitant release of alcohol product. The acyl-subtilisin then reacts with the exogenous nucleophile (an alcohol) to form another binary complex, which then yields the new ester and the free enzyme. In this study, subtilisin-catalyzed reaction 1 was kinetically investigated by varying the concentration of **1** in the range from 10 to 70 mM. An adherence to the aforementioned kinetic scheme was observed; e.g.,[10] linear dependence of the reciprocal initial rates vs reciprocal **1** concentrations was obtained. The slopes of these dependencies afford $k_{cat}/K_{M(alcohol)}$ (denoted as simply k_{cat}/K_M throughout this paper) values calculated as described in the Experimental Section. Note that these k_{cat}/K_M's are bimolecular rate constants for the reaction of the nucleophile (**1**) with butyryl-subtilisin.

(18) Throughout this study, we found that the general trends and conclusions made regarding enantioselectivities were *qualitatively* identical whether based on the initial rates of the enzymatic transesterification or on k_{cat}/K_M values for the individual enantiomers. Although the latter parameter is, generally speaking, preferable, it takes seven times as long to measure a k_{cat}/K_M compared to an initial rate. Consequently, given the large number of experiments conducted in this work, initial rates were used to calculate enantioselectivities in most instances. However, in critical experiments k_{cat}/K_M values were determined as well, thus leading to a seeming discrepancy in the enantioselectivity values reported (which, of course, *quantitatively* depend on which parameter was used to calculate them).

(19) For this alcohol, there is formally a reversal of enantioselectivity simply because chlorine has a higher order of priority than carbon in the Cahn–Ingold–Prelog system (Cahn, R. S.; Ingold, C.; Prelog, V. *Angew. Chem., Int. Ed. Engl.* **1966**, *5*, 385–415). To avoid confusion, **1**'s nomenclature was retained for 2-chloro-**1**.

Table III. Normalized Kinetic Components of Enantioselectivity of Subtilisin Carlsberg in Transesterification 1 in Anhydrous Solvents[a]

solvent	normalized[b] k_{cat}/K_M		enantioselect[c]
	S enant	*R* enant	
dioxane	0.22	0.0036	61
triethylamine	0.36	0.0075	48
tetrahydrofuran	0.32	0.0080	40
dimethylformamide	0.094	0.011	9
acetonitrile	0.11	0.037	3

[a] For reaction 1, conditions are outlined in footnote *a* to Table II; for the enzymatic transesterification between vinyl butyrate and benzyl alcohol, the conditions were the same except that the alcohol concentration range was from 10 to 50 mM. [b] Obtained by dividing the k_{cat}/K_M values from Table II by k_{cat}/K_M values for the transesterification involving benzyl alcohol instead of **1** in the same solvents. See text for details. [c] Obtained as in Table II.

because the solvent, expectedly,[10] greatly affects both $(k_{cat}/K_M)_S$ and $(k_{cat}/K_M)_R$. To subtract this general effect of the solvent on the reactivity of subtilisin,[10] we carried out a kinetic investigation of transesterification 1 where **1** was replaced with its achiral analogue, benzyl alcohol. The values of k_{cat}/K_M for this reaction were determined for five representative solvents out of the list in Table II, thereby providing a measure of the general influence of the solvent on subtilisin's reactivity. Subsequently, the k_{cat}/K_M values for (*S*)- and (*R*)-**1** from Table II were divided by k_{cat}/K_M values for benzyl alcohol in the same solvents. The resultant "normalized" k_{cat}/K_M data, given in Table III, indicate that most of the relaxation of enantioselectivity occurring in the series of solvents from top to bottom of the table are due to an increase in the reactivity of the *R* enantiomer: for example, upon transition from dioxane to acetonitrile $(k_{cat}/K_M)_R$ increases more than 10-fold, whereas $(k_{cat}/K_M)_S$ decreases only 2-fold (thereby leading to the overall 20-fold drop in enantioselectivity).

In order to explain why subtilisin's enantioselectivity depends on the reaction medium as shown in Table II, we attempted to correlate the enantioselectivity with basic physicochemical characteristics of the solvents. Among the latter,[20] such properties were considered as the dipole moment, dielectric constant, hydrophobicity,[21] Hildebrand solubility parameter, and several others. Only with the first two[22] was an unequivocal correlation found, i.e., the higher the dipole moment or dielectric constant, the lower subtilisin's enantioselectivity (Figure 2A and B). Questionable or no correlation was found with all other solvent parameters, as illustrated in Figure 2C for hydrophobicity, with which a correlation had been previously established[5] for another type of a subtilisin-catlyzed transesterification—that between a chiral ester (*N*-acetylalanine chloroethyl ester) and an achiral alcohol.

The uncovered correlations (Figure 2A and B) and the conclusions from Table III lead to the following hypothesis for the observed dependence of subtilisin's enantioselectivity on the solvent (Table II). A dominant feature of enzymes in anhydrous media is their high conformational rigidity.[4] This rigidity stems from stronger noncovalent interactions in such media than in water: all these interactions are essentially of the electrostatic origin[23] and hence their strength, according to Coulomb's law, is inversely dependent on the dielectric constant, which is higher for water than for almost all[20] organic solvents. The same reasoning suggests that enzymes should be much more rigid in anhydrous solvents of low dielectric constants (such as dioxane) than in those of high dielectric constants (such as acetonitrile). It was stated earlier that the marked enantioselectivity of subtilisin in dioxane is due to a poor fit of the phenyl group of **1**'s *R* enantiomer into the small binding pocket of the enzyme (Figure 1). Clearly, when the protein's flexibility increases (because of a higher dielectric constant of the solvent), the steric constraints become more forgiving, thereby allowing for a greater reactivity of the *R* enantiomer and, in turn, relaxed enantioselectivity.

Table IV. Effect of Additives on Enantioselectivity of Subtilisin Carlsberg in Transesterification 1 in Organic Solvents[a]

solvent	additive	enantioselect,[18] v_S/v_R
dioxane	none	50
dioxane	0.2% (v/v) water	18
dioxane	0.4% (v/v) water	14
dioxane	3.0% (v/v) formamide	5.9
acetonitrile	none	3.6
acetonitrile	0.4% (v/v) water	5.4

[a] The experimental conditions were the same as in Table I. No reaction was observed without enzyme. Even for the highest water content used (0.4%), during the time period required to measure the initial rates of enzymatic transesterification 1, less than 10% of vinyl butyrate present underwent enzymatic hydrolysis.

This hypothesis was verified experimentally. It predicts that any other means of enhancing subtilisin's conformational flexibility in dioxane should lower its enantioselectivity in reaction 1. Water acts as a lubricant in proteins,[24] and its addition to anhydrous solvents increases protein mobility in them.[25] Consequently, we examined the effect of addition of small amounts of water on subtilisin's enantioselectivity. As seen in Table IV, addition of as little as 0.2% water to dioxane indeed lowers the enzyme enantioselectivity from 50 to 18 and still further to 14 when the water content is doubled. Significantly, in acetonitrile, 0.4% water not only does not reduce the enantioselectivity but, in fact, somewhat raises it (the last two lines in Table IV).

Since water's role as a molecular lubricant in proteins[24] is due to its ability to form multiple hydrogen bombs, other solvents mimicking water in this regard can, at least partially, substitute for it in organic solvent systems.[26] Therefore we added one such solvent,[26] formamide, to dioxane and found (Table IV) that 3% formamide indeed reduces subtilisin's enantioselectivity in dioxane more than 8-fold.

It was essential to confirm that the observed relaxation of enantioselectivity of subtilisin in dioxane brought about by water and formamide is due to a higher reactivity of the *R* enantiomer (as our hypothesis predicts) rather than a lower reactivity of its *S* counterpart. One can see in Table V that again, as in Table II, such a distinction is difficult to make. Hence, as with Table III, we normalized v_S and v_R by dividing them by the initial rates of the transesterification reaction involving **1**'s achiral analogue, benzyl alcohol. The resultant normalized data (Table V) are very clear in demonstrating that only the *R* enantiomer is affected (activated) by the additives.

In addition, according to what the above-described hypothesis would predict, replacement of **1** as the nucleophile with other alcohols in transesterification 1 results in parallel changes in enantioselectivity in different solvents (Table I) regardless of

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(22) It is hardly surprising that the dipole moment and dielectric constant act in a similar manner because they are intimately related via the Debye equation (Exner, O. *Dipole Moments in Organic Chemistry*; Georg Thieme Publishers: Stuttgart, Germany, 1975; Chapter 1). Note that a satisfactory correlation was also found between subtilisin's enantioselectivity and the Kosower Z or $E_T(30)$ solvent polarity parameter[20] (this parameter has been successfully used in a recent elegant study (Smithrud, D. B.; Diederich, F. *J. Am. Chem. Soc.* **1990**, *112*, 339–343) to rationalize the strength of host–guest complexation in different organic solvents). However, this solvent polarity parameter was not used in our analysis because it usually correlates with dielectric constant and dipole moment[20] but, in contrast to the latter two, is purely empirical and thus does not directly afford mechanistic interpretations.

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Table V. Effect of Additives on Kinetic Components of Enantioselectivity of Subtilisin Carlsberg in Transesterification 1 in Dioxane[a]

additive	v_S, mM/h	v_R, mM/h	normalized[b] v_S	normalized[b] v_R	enantioselect,[18] v_S/v_R
none	1.1	0.022	0.19	0.0038	50
0.4% (v/v) water	1.3	0.092	0.19	0.014	14
3% (v/v) formamide	0.077	0.013	0.25	0.043	6

[a] For experimental conditions, see Table IV. The concentration of benzyl alcohol was the same (10 mM) as that of **1**. [b] Calculated by dividing the corresponding v_S and v_R values by the initial rates of the enzymatic transesterification with benzyl alcohol in the same solvents.

Table VI. Enantioselectivity of Subtilisin BPN′ in Transesterification 1 in Various Anhydrous Solvents[a]

solvent	v_S, mM/h	v_R, mM/h	enantioselect,[18] v_S/v_R
dioxane	0.77	0.11	7.0
benzene	0.096	0.014	6.9
tetrahydrofuran	0.64	0.12	5.3
nitromethane	0.20	0.11	1.8
acetonitrile	0.31	0.20	1.6

[a] The conditions were the same as those for subtilisin Carlsberg described in Table I.

Table VII. Enantioselectivity of Porcine Pancreatic Lipase in Transesterification 1 in Various Anhydrous Solvents[a]

solvent	v_R, mM/h	v_S, mM/h	enantioselect,[18] v_R/v_S
nitromethane	9.7	0.13	75
dimethylformamide	2.3	0.038	61
triethylamine	4.2	0.099	42
tert-amyl alcohol	6.9	0.20	35
butanone	8.6	0.32	27
acetonitrile	14	0.64	22
benzene	10	0.67	15
cyclohexane	43	3.3	13
decane	5.5	0.85	6

[a] The conditions were the same as in Table I, except that the concentration of **1** was 100 mM and that of the enzyme was 100 mg/mL.

whether subtilisin's enantioselectivity with **1** in them was high (dioxane), low (acetonitrile), or intermediate (tetrahydrofuran).

We decided to explore whether the phenomena uncovered for subtilisin Carlsberg will also exist with a closely related enzyme, subtilisin BPN′ (protease from *Bacillus amyloliquefaciens*).[27] Table VI presents the kinetic data on the enantioselectivity of this enzyme in transesterification 1. As in the case of subtilisin Carlsberg (Table II), the enantioselectivity of subtilisin BPN′ both significantly depends on the reaction medium and correlates with solvents' dielectric constants and dipole moments. That the range of enantioselectivity (i.e., the maximal enantiodiscrimination) for subtilisin BPN′ is much lower than for subtilisin Carlsberg suggests that for the former protease either the small binding pocket is more spacious or the large binding pocket is more cramped (Figure 1). Interestingly, there is a similarity in the effect of added water on enantioselectivity of the two subtilisins: at 0.4% water in dioxane, the enantioselectivity of the BPN′ enzyme is more than a third lower than in the anhydrous solvent, while in the case of acetonitrile this addition of water makes no difference.

In summary, this study demonstrates that a synthetically useful process, an enzymatic resolution of racemic alcohols, can be profoundly controlled by the solvent. A physicochemical rationale was developed and confirmed experimentally that mechanistically explains this phenomenon. This rationale has a proven predictive power: for instance, some of the solvents in Table II were added after our hypothesis had been formulated and the enantioselectivity values obtained turned out to be in good agreement with those predicted on the basis of dielectric constants or dipole moments. In addition, when 1,4-dioxane was replaced as the reaction medium with its isomer 1,3-dioxane, the enantioselectivity (v_S/v_R) of subtilisin Carlsberg in transesterification 1 dropped from 50 to 25; this result is quite indicative because the two dioxanes have the same log P value[21] but very different dipole moment values (0.45 and 2.13 D,[20] respectively).

The enantioselectivity of subtilisin in transesterification 1 correlates with the dielectric constant and dipole moment of the solvent but much less so with its hydrophobicity (with dioxane being a particularly conspicuous exception); this observation is interpreted in terms of the effect of the solvent on protein conformational mobility. In contrast, the enantioselectivity of this enzyme in the transesterification of *N*-acetylalanine chloroethyl ester and the achiral alcohol propanol correlates well with the solvent's hydrophobicity; this effect is interpreted in terms of partitioning of the water, associated with the binding site, between the enzyme and the solvent.[5] This difference is presumably due to the fact that the chiral components of the two transesterifications in question, an ester of *N*-acetylalanine and a secondary alcohol, bind to the two distinct areas of the enzyme's binding site. The loci of binding of the substrate ester and the nucleophile are indeed known to be distinct for serine proteases.[16] The former locus is utilized in water and is responsible for the L enantioselectivity of proteolytic enzymes.[16] The alcohol-binding site (Figure 1), on the other hand, has no obvious biological function and thus should be fortuitous. One would expect that it may vary depending on the structure of the nucleophile. Indeed, we found[6] that when chiral amines were used (instead of **1**) as nucleophiles, subtilisin's enantioselectivity again was greatly affected by the solvent but in a way different from that delineated in the present study (no correlation with either dipole moment or dielectric constant). The challenge now is to elucidate mechanistically all the possible modes of the effect of the reaction medium on subtilisin's enantioselectivity and reconcile them with each other. Also, one wonders whether the solvent can reverse enzyme enantioselectivity, i.e., make *S* enantiomer preferred in one solvent and *R* in another.

Finally, it should be emphasized that solvent control of enantioselectivity is not limited to subtilisin. For example, our preliminary studies with porcine pancreatic lipase (a widely used asymmetric catalyst in organic solvents[9]) show that its enantioselectivity in transesterification 1 also markedly depends on the solvent (Table VII). In this case, enantioselectivity (which, interestingly, is opposite to that of the subtilisins) seems to correlate with neither dipole moment nor hydrophobicity; work is underway to analyze this phenomenon mechanistically. On a practical note, these lipase data, combined with those for subtilisin, underscore the usefulness and generality of incorporating the solvent optimization step in the overall strategy of the biocatalytic asymmetric resolutions. While in nearly all previous investigations of this sort the solvent has been selected purely on the basis of its effect on enzyme activity and substrate solubility,[9] a consideration of its influence on enzyme enantioselectivity is now clearly prescribed. In the absence of a universal and comprehensive predictive model, alluded to in the Introduction, one should at least undertake a solvent screening along with a conventional[1] biocatalyst screening.

Experimental Section

Enzymes. Subtilisins Carlsberg and BPN′ (serine proteases from *Bacillus licheniformis* and *Bacillus amyloliquefaciens*, EC 3.4.21.14) and porcine pancreatic lipase (EC 3.1.1.3) were all purchased from Sigma Chemical Co. The concentration of the active centers in subtilisin Carlsberg, determined by spectrophotometric titration with *N*-*trans*-cinnamoylimidazole,[28] was found to be 54%. The subtilisins were prepared[10] by dissolving them (5 mg/mL) in 20 mM aqueous potassium phosphate buffer (pH 7.8), followed by lyophilization. It was observed

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that the catalytic activity of the subtilisins in transesterification 1 (and probably other reactions in organic solvents) strongly depended on the water content of the lyophilized enzymes. Hence, in order to keep the water content of different subtilisin preparations constant, the lyophilized samples of the subtilisins were placed in a desiccator containing a vessel with the saturated aqueous solution of LiCl. The desiccator was evacuated and stored at 7 °C for at least 48 h before the enzymes were used. Under these conditions the enzymes, exposed to a constant humidity of 11%,[29] exhibit reproducible activities in organic solvents over the period of at least 7 days. Porcine pancreatic lipase (specific activity of 11 triacetin units/mg of solid) was used as a catalyst in organic solvents directly without any pretreatment.[30]

Chemicals and Solvents. All the alcohols used in this study, including the individual enantiomers of chiral alcohols, were obtained from commercial suppliers [except for the *R* and *S* enantiomers of *sec*-(2-naphthyl)ethyl alcohol, which were obtained by the enzymatic resolution of the racemate[8]] and were of analytical grade or purer. Vinyl butyrate (99%+ pure by gas chromatography) was purchased from American Tokyo Kasei Co. Butyryl esters of chiral alcohols, used as standards in gas chromatographic analyses, were synthesized according to the classical methodology.[31] Organic solvents employed in this work were either purchased in the anhydrous form (in Aldrich Sure/Seal bottles, water content below 0.005%) or dehydrated by shaking with 3-Å molecular sieves (Linde)[10] to bring the water content[32] below 0.01%. Triethylamine was purified by using the literature procedure.[33]

Kinetic Measurements. In a typical experiment, a powdered enzyme sample (prepared as described above) was placed in a 7-mL screw-cap scintillation vial, followed by an addition of 1 mL of a solvent containing vinyl butyrate and an alcohol. Then the vial was closed, subjected to a 5-s sonication (to homogenize the suspension), placed in a controlled-temperature shaker, and shaken at 45 °C and 300 rpm. Periodically, 0.5-μL aliquots were withdrawn and assayed by gas chromatography (10-m HP-5 capillary column coated with 5% phenyl-/95% methylsilicone gum). The reaction rates were determined on the basis of the increase in the concentration of the product butyryl esters (e.g., reaction 1) as a function of time (five to seven data points were usually collected).

Kinetic Calculations. The values of V/K_M (where K_M stands for the Michaelis constant for the alcohol in the enzymatic transesterification) were determined on the basis of the dependencies of the initial rates of the enzymatic reactions on the alcohol concentrations (typically, seven data points were obtained). Nonlinear regression analysis using the software program, Enzfitter, written by R. J. Leatherbarrow and distributed by Elsevier-Biosoft, was employed in all calculations. The enzyme molarity (needed to convert V/K_M into k_{cat}/K_M) was determined from the molecular weight and weight concentrations (mg/mL) of subtilisin Carlsberg used in transesterifications and the following correction factors:[10] the aforementioned 54% purity of the enzyme, the fact that only 68% of all subtilisin molecules are catalytically competent in organic solvents, and the presence of 40% (w/w) of potassium phosphate salts in the lyophilized enzyme samples. Note that any inaccuracies arising from these calculations would affect $(k_{cat}/K_M)_S$ and $(k_{cat}/K_M)_R$ to the same extent and thus would be inconsequential for our conclusions.

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Communications to the Editor

Electronic Structure of *trans*-Dioxorhenium(VI)

John C. Brewer,[†] H. Holden Thorp,[‡,§] Kirby M. Slagle,[†] Gary W. Brudvig,*[,‡] and Harry B. Gray*[,†]

Contribution No. 8231 from the Beckman Institute California Institute of Technology Pasadena, California 91125 Department of Chemistry, Yale University New Haven, Connecticut 06511
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Complexes containing d^2 *trans*-dioxometal units exhibit rich electrochemistry[1-3] and photochemistry.[2,4-6] By employing strongly basic ancillary ligands, we have succeeded in isolating the first d^1 *trans*-dioxo complex, *trans*-$[ReO_2(dmap)_4](PF_6)_2$ [dmap = 4-(dimethylamino)pyridine].[6] Analysis of the structure of the complex cation shows that there is considerable shortening (0.04 Å) in the Re-N bond lengths relative to Re(V) analogues,[6,7] but the Re-oxo bond lengths are virtually the same in both oxidation states (Figure 1A).[8] The structural data confirm that an electron is removed from the d_{xy} orbital upon oxidation of Re(V), as predicted by the standard ligand field (LF) model for axially compressed metal-oxo systems (Figure 1B).[9-11] In order to examine the electronic structure of the *trans*-dioxo framework more closely, we have measured and analyzed the EPR spectrum of *trans*-$[ReO_2(dmap)_4](PF_6)_2$.

The EPR spectra of d^1 Re(VI) species often are hard to interpret because of the extremely large Re hyperfine and quadrupole coupling constants associated with tetragonal geometries of this ion.[12-15] As a result, the spectra exhibit (1) variations in band

[†] Beckman Institute, California Institute of Technology.
[‡] Yale University.
[§] Present address: Department of Chemistry, North Carolina State University, Raleigh, NC 27695-8204.

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